Atypical ubiquitin chains: new molecular signals

'Protein Modifications: Beyond the Usual Suspects' Review Series

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Ubiquitin (Ub) is a small protein modifier that regulates many biological processes, including gene transcription, cell-cycle progression, DNA repair, apoptosis, virus budding and receptor endocytosis. Ub can be conjugated to target proteins either as a monomer or as Ub chains that vary in length and linkage type. The various types of Ub modification are linked to distinct physiological functions in cells. MonoUb, for example, regulates DNA repair and receptor endocytosis, whereas lysine 48-linked Ub chains label proteins for proteasomal degradation. More recently, the importance of chains conjugated through the other six lysines in Ub, known as atypical Ub chains, has been revealed. Atypical chains can be homotypic, sequentially using the same lysine residue in Ub for conjugation; mixed-linkage, utilizing several distinct lysines to connect consecutive Ub moieties; or heterologous, connecting Ub with other Ub-like modifiers. Here, we describe recent progress in the understanding of atypical Ub chain assembly and their recognition by Ub-binding domains, and we discuss further their functional roles *in vivo*.

Introduction

Protein modification by ubiquitin (Ub)—a process known as ubiquitination or ubiquitylation—is involved in the regulation of numerous cellular functions, including protein stability, cell-cycle progression, gene transcription, receptor transport, immune responses and viral infection (Haglund & Dikic, 2005; Mukhopadhyay & Riezman, 2007). In general, these modifications are controlled by a three-step reaction initialized by E1-mediated activation of Ub, followed by

conjugation to an E2 enzyme and finally substrate targeting by an E3 Ub ligase (Hershko & Ciechanover, 1998; Pickart & Eddins, 2003). Similar to the process of phosphorylation and dephosphorylation, ubiquitination is also reversible, and the cleavage of Ub from substrates is carried out by specific deubiquitinating enzymes (DUBs; Hochstrasser, 1995).

Ubiquitin is one of the most versatile molecular signals in the cell because of its ability to modify substrate proteins in its monomeric form (monoubiquitination) or to be conjugated to preceding Ub moieties and consequently form many types of Ub chain (polyubiquitination; Haglund & Dikic, 2005; Welchman *et al*, 2005). Monoubiquitination has been shown to control numerous cellular processes such as receptor transport, viral budding and DNA repair (Hicke, 2001; Di Fiore *et al*, 2003; Haglund *et al*, 2003), whereas Ub chains that form through their lysine (Lys) residues at position 48 (Lys 48) are known to regulate protein stability (Hershko & Ciechanover, 1998). Ub can also be conjugated through other lysine residues on its surface (Lys 6, Lys 11, Lys 27, Lys 29, Lys 33 and Lys 63), forming Ub chains of various lengths and shapes *in vitro* and *in vivo* (Johnson *et al*, 1995; Peng *et al*, 2003; Kim *et al*, 2007). Different Ub linkages result in various conformations of Ub chain and create a range of molecular signals in the cell.

In addition to Ub itself, many ubiquitin-like (Ubl) proteins have been identified, including small Ub-like modifier (SUMO), interferon-stimulated gene 15 (ISG15), autophagy 8 (ATG8) and neural precursor cell expressed, developmentally downregulated 8 (NEDD8; Kerscher *et al*, 2006). Ubls have a similar structural-fold to Ub, but use specific conjugation machineries and are recognized by distinct Ubl-binding domains. So far, SUMO2/3 and NEDD8 are the only Ubls known to participate in chain formation (Tatham *et al*, 2001; Knipscheer *et al*, 2007; Matic *et al*, 2008; Xirodimas *et al*, 2008). Ubls are implicated in the regulation of many cellular processes such as gene transcription, signal transduction, autophagy and cell-cycle control (Kerscher *et al*, 2006).

The conserved ubiquitin β-grasp fold has had numerous functions during evolution and is used, for example, as a protein domain integrated in the coding sequence of several cellular proteins. These ubiquitin-like domains (ULDs) adopt a Ub-like fold within the tertiary structure of the host protein (Tanaka *et al*, 1990; Hartmann-Petersen & Gordon, 2004; Ikeda *et al*, 2007). ULDs can intrinsically

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regulate the conformational status of the host protein, as well as participate in the reorganization of protein complexes (Tanaka *et al*, 1990; Hartmann-Petersen & Gordon, 2004; Ikeda *et al*, 2007).

Classification of atypical ubiquitin chains

Atypical Ub chains include all variations of multimeric Ub structure with the exception of classical Lys 48 polyubiquitination, which is considered to be a typical Ub chain based on its original discovery as a destruction tag for proteasomal degradation. To distinguish various types of atypical Ub chain, we have divided them into several classes (Fig 1). Chains formed by the conjugation of a single type of lysine residue in sequential Ub molecules are homotypic, whereas those assembled through several distinct lysines in the Ub monomers are mixed-linkage chains. As a consequence of using divergent lysines for conjugation, mixed-linkage Ub chains form bifurcations, such as chains containing two different types of linkage—Lys 6/11, Lys 27/29, Lys 29/48 or Lys 29/33 (Kim *et al*, 2007). Moreover, the integration of other Ubl modifiers, such as SUMO and NEDD8, into Ub chains gives rise to heterologous Ub chains; so far, the heterologous chains are the least studied. Finally, many monoUbs attached to a substrate, when packed spatially in close proximity, can be considered as atypical Ub signals with acquired Ub multivalency.

Assembly of various types of ubiquitin chain

During ubiquitination, Ub is initially activated by an ATP-dependent E1 enzyme before it is passed to one of several distinct Ub-conjugating enzymes (E2s). The E2 subsequently acts to either transfer Ub to a HECT (homologous to the E6AP carboxyl terminus)-type Ub ligase (E3), or to catalyse substrate ubiquitination in conjunction with RING (really interesting new gene)-type or U-box E3 enzymes. An exception to this is the process of coupled monoubiquitination, through which E2 enzymes can catalyse the ubiquitination of Ub-binding domain (UBD)-containing proteins independently of E3 (Hoeller *et al*, 2006, 2007).

Ubiquitin ligases seem to be the crucial determinants of substrate selection and are also considered to be important components in controlling Ub-chain formation on a substrate. For example, different HECT-type E3s can specify both the linkage of a Ub chain and the process of its assembly. E6AP can form a Lys 48-linked chain on its active cysteine residue (Scheffner & Whitaker, 2003), whereas KIAA10 catalyses Lys 48- and Lys 29-linked chains as free entities (Wang *et al*, 2006). In budding yeast, the Ub ligase Rsp5 has been shown to modify specific substrates either with monoUb, Lys 48- or Lys 63-linked chains (Kee *et al*, 2005). Correspondingly, the mammalian RING-type ligase CBL mediates multiple monoubiquitination and Lys 11-, Lys 48- and Lys 63-oligoubiquitination of activated epidermal growth factor receptor (EGFR; Haglund *et al*, 2003; Mosesson *et al*, 2003; Huang *et al*, 2006), as well as Lys 48-linked polyubiquitination of SRC kinases (Thien & Langdon, 2005).

Besides the E3s, E2 enzymes can also be structured to control lysine specificity during Ub chain assembly. Although most E2s are designed to ubiquitinate substrates with no inherent selectivity for a specific acceptor lysine residue, some stimulate Ub chain formation linked through a defined lysine in Ub. Structural studies of the MMS2–UBC13 complex have given rise to a model describing the specific elongation of Lys 63-linked chains. In this model, the catalytically active E2 enzyme UBC13, when conjugated with Ub,
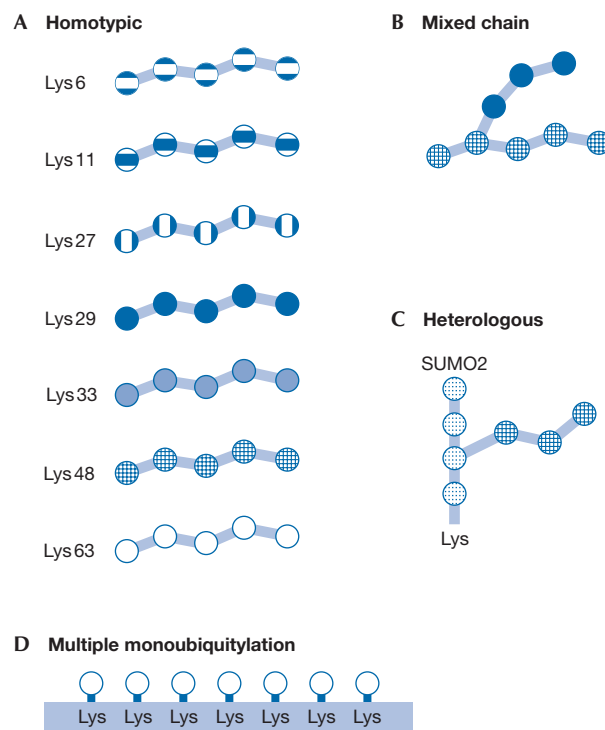

Fig 1 | A schematic model of possible ubiquitin chain formations on a target protein. **(A)** Homotypic atypical and typical chains, such as lysine 6 (Lys 6)-, Lys 11-, Lys 27-, Lys 29-, Lys 33-, Lys 48- and Lys 63-linked ubiquitin (Ub) chains. **(B)** Mixed-linkage atypical chains are formed by the use of different lysines for sequential Ub conjugation, leading to the formation of bifurcated chains, for example, Lys 48/Lys 29 forks. **(C)** Heterologous chains are formed between Ub and ubiquitin-like proteins, for example the small ubiquitin-like modifier (SUMO) 2. **(D)** Multiple monoubiquitination moieties represent a subtype of multivalent chain-like Ub signals owing to the spatial organization of multiple monoUb molecules attached to the substrate.

binds to MMS2, a pseudo-E2 enzyme with the ability to bind an acceptor Ub. As a consequence of this interaction, the acceptor Ub on MMS2 is positioned in such a way that only Lys 63 is accessible for Ub transfer from UBC13, hence forming Lys 63-linked chains (VanDemark *et al*, 2001). Conversely, other E2s, such as UBCH5, can catalyse the formation of Ub chains that lack specificity for any lysine residue of Ub (Brzovic *et al*, 2006).

Individual E3 ligases commonly interact with several E2 enzymes to correctly modify target substrates. In the case of the E3 ligase breast cancer susceptibility gene 1 (BRCA1), interaction with the E2s UBCH6, UBE2E2, UBCM2 and UBE2W supports mono-ubiquitination, whereas MMS2–UBC13 and UBE2K catalyse Lys 63- or Lys 48-linked chain formation on BRCA1, respectively (Christensen *et al*, 2007). Moreover, certain pairs of E2s and E3s can synthesize Ub chains containing different isopeptide linkages (Kim *et al*, 2007; Windheim *et al*, 2008). The E2 UBCH5, together with the E3 ligases CHIP and MDM2, induce the assembly of homotypic chains by using all seven possible linkages, as well as mixed-linkage chains joined through adjacent lysines at Lys 6/11, Lys 27/29 or Lys 29/33 (Kim *et al*, 2007). By contrast, in the presence of UBCH13/UEV1A

and UBCH1 the same E3s can induce Lys 63 and Lys 48 chains, respectively. Thus, various combinations of E2 and E3 enzymes can direct the synthesis of diverse types of atypical Ub chain during *in vitro* reactions (Sidebar A). It is likely that additional factors, including subcellular compartmentalization of selected E2 and E3 pairs, are detrimental to the formation of some Ub chains *in vivo*.

The original idea that Ub chains are assembled by the sequential addition of Ub monomers to a lysine residue in the target protein is now challenged by evidence indicating that Ub polymers can be formed on E2/E3 enzymes before target substrate conjugation (Wu-Baer *et al*, 2003; Ben-Saadon *et al*, 2006; Li *et al*, 2007). E3 enzymes often form different types of Ub chain at their active sites (Hochstrasser, 2006), an activity that was originally thought to be a mechanism for controlling the abundance of the ligases by marking them for degradation (Brown *et al*, 2002). However, self-ubiquitination through mixed-linkage Ub chains can have a regulatory role, as in the case of the Polycomb E3 RING-type ligases Ring1B and BMI1 (Ben-Saadon *et al*, 2006). Moreover, it was shown that Lys 63-linked self-ubiquitination of TNF receptor-associated factor 6 (TRAF6) is crucial for IκB kinase complex activation *in vivo* (Lamothe *et al*, 2007). E2 enzymes can also assemble divergent Ub chains at their active sites, which can then be transferred to a substrate. One such example is the UBE2G2 E2 enzyme that, together with the E3 ligase GP78, mediates substrate ubiquitination by using Ub chains pre-assembled at the catalytic cysteine of UBE2G2 (Li *et al*, 2007). In addition, Ub can be transferred from one E2 to another, thus forming diUb chains presumably owing to E2 dimerization. Interestingly, dimerization of CDC34 (cell division cycle 34), which is induced by thioester formation between Ub and CDC34, is essential for the synthesis of Lys 48-linked Ub chains by this enzyme (Varelas *et al*, 2003).

Quantitative analyses of atypical chains

The analysis of the formation and dynamic changes of atypical Ub chains represents the main bottleneck in understanding the structural complexity and functional diversity of these modifications *in vivo*. Most of the collected data are derived from biochemical experiments performed with purified proteins and synthetic Ub chains or from studies using transfection of tagged versions of lysine-to-arginine mutant forms of Ub in cells. The problem is that there are no controlled comparisons between different experimental conditions—for example, extent of protein overexpression or influence of endogenous Ub on the specific lysine-to-arginine mutants—and current conclusions often rely on several assumptions, which do not always represent physiological Ub modifications *in vivo*. In addition, antibodies with preferences for Ub chains are not sufficiently sensitive and specific for comparative analyses of distinct Ub modifications in cells.

The most powerful analytical method for measuring global changes in Ub linkages is the use of mass spectrometry (Kirkpatrick

et al, 2005). The first large-scale analysis of all ubiquitinated proteins in yeast expressing epitope-tagged Ub identified 1,075 candidate substrates (Peng *et al*, 2003). All seven lysine residues have been found to participate in the formation of Ub–Ub linkages, with a relative abundance order of Lys 48 > Lys 11 and Lys 63 >> Lys 6, Lys 27, Lys 29 and Lys 33. Ub peptides with mixed linkages of Lys 29 and Lys 33 were also identified, although in a low proportion compared with conjugated monoUb or different types of homotypic chain (Peng *et al*, 2003). Such quantitative data are valuable, but not complete owing to intrinsic technical limitations. For example, mass spectrometry analyses are inherently biased towards more abundant species and might give an incorrect impression of the complexity of less abundant Ub chains. As pointed out by Kirkpatrick and colleagues, the absence of evidence should not necessarily be construed as evidence of absence (Kirkpatrick *et al*, 2005).

Recent development of the Ub-AQUA (absolute quantification of Ub) method has provided a new tool for more precise quantitative characterization of the various forms of Ub chain (Kirkpatrick *et al*, 2006). In the AQUA method, all seven ubiquitinated -GG peptides are labelled with a stable isotope and then quantified as internal standards, which can be distinguished in a mass spectrometer. Quantitative analysis of *in vitro* ubiquitinated cyclin B1 revealed complex chain topology, including Ub linked through lysine residues (Lys 63, Lys 11 and Lys 48). Interestingly, even in the absence of Lys 48 the remaining Ub chains were able to mediate degradation of cyclin B1 in the proteasome (Kirkpatrick *et al*, 2006). In accordance with this observation, homotypic Ub chains linked through Lys 63, but not mixed-linkage chains, can mediate proteasomal degradation *in vitro* as rapidly as Lys 48-linked Ub chains (Kim *et al*, 2007).

However, a significant obstacle in quantitative *in vivo* analysis of Ub chains and their recognition by Ub-binding proteins is the low abundance of substrates, the modifications of which vary markedly under certain conditions in cells. Such modified proteins often escape detection by current proteomics technologies, indicating the need for rapid development of more sensitive proteomics platforms.

Recognition of ubiquitin chains by ubiquitin-binding domains

More than 20 families of Ub-binding domain (UBD), which bind to monoUb and Ub chains, have been identified (Hicke *et al*, 2005; Hurley *et al*, 2006; Husnjak *et al*, 2008; Iha *et al*, 2008; Schreiner *et al*, 2008; Wagner *et al*, 2008). The *in vitro* binding affinity between a UBD and Ub is usually within $K_d = 10–500\,\mu M$, which is relatively low for a physiological interaction. Conversely, the level of free Ub in cells is fairly high—2 μM in muscle tissues and 10–20 μM in different cultured cells (Haas & Bright, 1985; Haas, 1988; Riley *et al*, 1988)—which raises the question how UBD-containing proteins can selectively bind to conjugated Ub and not to free Ub. It is likely that physiological Ub–UBD interactions are regulated by the avidity of binding, which might be mediated through multivalent interactions between ubiquitinated substrates and Ub-binding proteins. This can be achieved in several ways, including multiplication of UBDs in adaptor proteins, oligomerization of Ub-binding proteins, secondary Ub-independent interactions brought about in signalling complexes or by an accumulation of interacting partners in defined cellular compartments. For example, the Ub-interacting motif (UIM) domains in EPS15, epsin and HRS bind to monoUb with relatively low affinities ($K_d > 100\,\mu M$; Hurley *et al*, 2006). However, these interactions are physiologically relevant for the sorting of ubiquitinated

Fig 2 | Structural models indicating differences between Lys 48 and Lys 63 signals. ▶
(A) Lysine 48 (Lys 48)-linked di-ubiquitin (diUb; left panel) and Lys 63-linked
diUb (right panel) show two different conformations, which indicate distinct
binding properties for interaction with ubiquitin-binding domains (UBDs).
Structures of other atypical chains have not yet been determined.
(B) Conformational differences between alternatively linked polyubiquitin
chains allow them to interact with UBDs in distinct binding modes: a sandwich-
like mode (left panel) and as multiple independent UBD-binding sites (right
panel). Shown here are structural models of the complexes of Lys 48- and
Lys 63-linked diUb (left and right panels, respectively) with the UBA2 domain
of HR23a. The distal and proximal Ubs are shown in surface representation,
coloured light blue and green, respectively; the UBA domains are shown as
ribbon. The hydrophobic patch residues (Leu 8–Ile 44–Val70) on the Ub surface
are painted blue. **(C)** Lys 48-linked diUb can bind to UBDs at several binding
sites, as observed in the case of the UBA domain of ubiquilin-1 (PLIC-1). UBA,
ubiquitin-associated.

cargoes in the endosome, probably as a result of the oligomeriza-
tion of endosomal Ub receptors and the presence of many mono-
Ubs on transport cargoes (Hicke, 2001; Di Fiore *et al*, 2003; Haglund
et al, 2003). Interestingly, the UIM domain of HRS folds into a
double-sided α-helix with the ability to simultaneously bind to two
Ub molecules, one on each side (Hirano *et al*, 2006).

One of the important challenges in the field is to understand
why and how some UBDs preferentially bind to Ub chains with
linkage specificity, whereas others are more or less promiscuous
with regard to linkage type. Initial clues have come from the reali-
zation that Ub chains have different conformations that provide
specific surfaces for interactions with UBDs (Fig 2). For exam-
ple, Lys 48-linked Ub polymers adopt a compact conformation,
whereas Lys 63 diUb acquires an extended shape, similar to a lin-
ear chain (Varadan *et al*, 2002, 2004; Fig 2A). In a comprehensive
study involving more than 30 Ub-associated (UBA) domains, it
was shown that one subgroup preferentially binds to Lys 48-linked
chains, a second subgroup primarily recognizes Lys 63-linked
chains and a third subgroup binds to Ub chains without any pref-
erence for linkage specificity (Raasi *et al*, 2005). Interestingly, all
of the UBAs that recognize Ub chains also bind to monoUb (Raasi
& Pickart, 2002). Conformational studies of the UBA domains in
HR23a and MUD1 in solution bound to Lys 48-linked diUb, indi-
cating that UBA domains bind between the two monoUb molec-
ules in a 'sandwich-like' manner (Trempe *et al*, 2005; Varadan
et al, 2005; Fig 2B, left panel). By contrast, the open conformation
of the Lys 63-linked diUb allows interaction with the human HR23a
UBA domain independent of additional interactions, similar to the
binding to linear monoUb (Varadan *et al*, 2004; Fig 2B, right panel).
The UBA domain of ubiquilin 1 (PLIC-1) binds to Lys 48-linked diUb
by using independent surfaces of each of the Ub molecules (Fig 2C),
which is distinct from the mode of binding of the UBA2 domain of
HR23a (Fig 2B, left panel; Zhang *et al*, 2008). A similar situation to
the UBA domain of PLIC-1 is observed with a UIM of S5a, which
also recognizes both Lys 48- and Lys 63-linked chains (Haririnia
et al, 2007).

More recently, RPN13/ARM1 was identified as a new Ub recep-
tor at the proteasome and shown to stoichiometrically bind to Lys 48-
linked diUb through its pleckstrin-like receptor for ubiquitin (Pru)
domain (Husnjak *et al*, 2008; Schreiner *et al*, 2008). Fluorescence
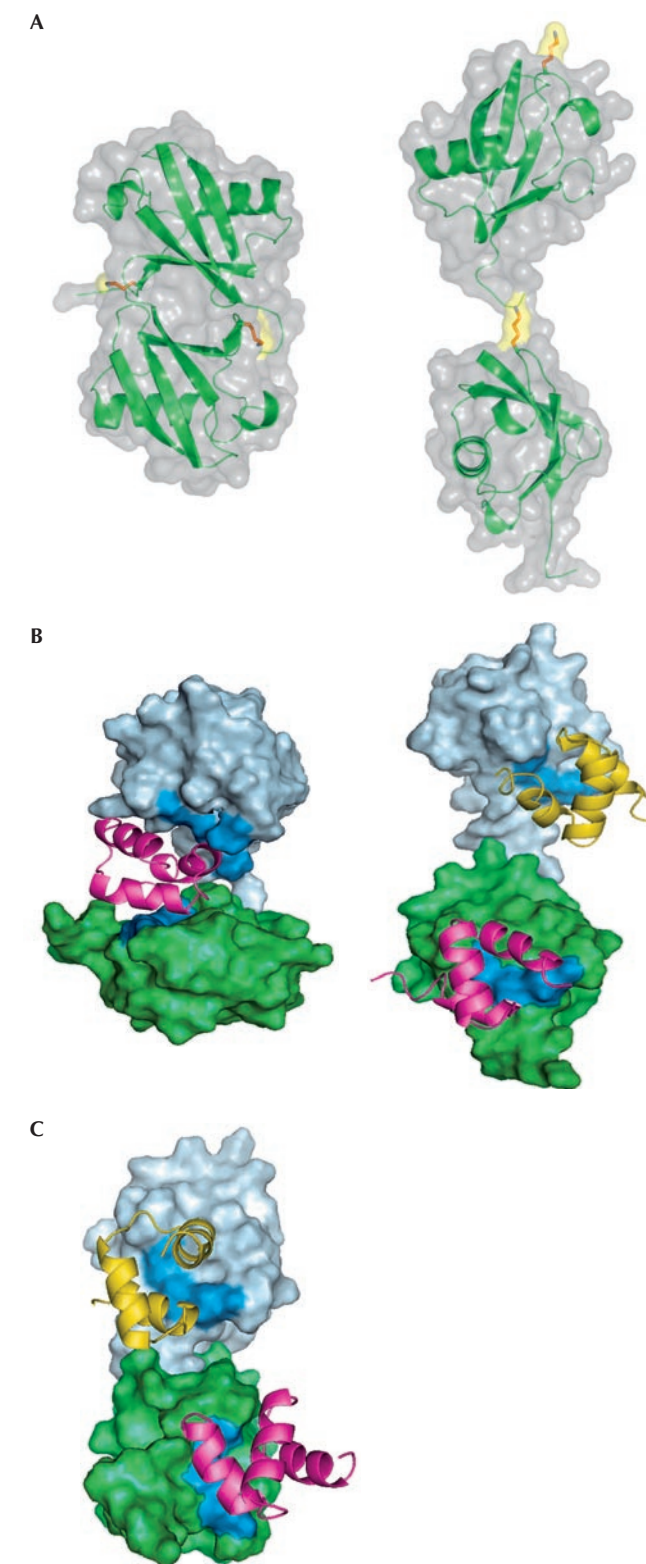
spectroscopy analysis revealed that the Pru domain binds to Lys 48-
linked diUb with a K_d of 90 nM and monoUb with a K_d of 300 nM,
which is the highest affinity observed among known UBDs (Husnjak
et al, 2008). In this particular case, the loops of the Pru domain,

rather than secondary structural elements, create a large interaction surface with Ub (1,256 Å^2), thus providing the rationale for the observed high affinity.

Recent studies have reported the characterization of new UBDs that show specificity for Lys 63-linked chains *in vivo*. A UBD found in NEMO, optineurin and A20 binding inhibitor of NF-κB activation (ABIN) proteins—commonly named UBAN (UBD in ABIN proteins and NEMO)—is involved in the regulation of NF-κB signalling pathways (Ea *et al*, 2006; Wu *et al*, 2006; Zhu *et al*, 2007; Bloor *et al*, 2008; Wagner *et al*, 2008). In all these cases, the UBAN domain binds preferentially to Lys 63-linked chains or linear Ub polymers, with the exception of the UBAN domain in ABIN3, which was found to interact with monoUb as potently as with Ub chains (Wagner *et al*, 2008). Nuclear magnetic resonance analysis of the UBAN domain in ABIN1 clarified that UBAN recognizes an extended surface of Ub, suggesting that the UBAN domain has an elongated shape that prefers the linear conformation of Lys 63 chains (Wagner *et al*, 2008). In addition, the Ub-binding zinc-finger (UBZ) domain of the adaptor protein TAX1BP1 was recently shown to target Lys 63-ubiquitinated forms of TRAF6 and the receptor-interacting protein RIP in tumour necrosis factor-α (TNFα) and interleukin-1 (IL-1)-treated cells, respectively (Iha *et al*, 2008). However, *in vitro* TAX1BP1 can bind to monoUb, Lys 63- and Lys 48-linked chains. TAX1BP1 is constitutively associated with the deubiquitinating enzyme A20, which removes Ub chains from interacting proteins leading to the inhibition of NF-κB signalling (Iha *et al*, 2008).

Physiological roles of atypical ubiquitin chains

Although all lysine residues in Ub have been shown to participate in chain formation *in vivo* under different conditions, only a few studies have addressed their biological significance in cells. The most-studied of the atypical Ub chains is the Lys 63-linked chain, which has a crucial role in signal transduction through the NF-κB pathway, as well as in receptor endocytosis and DNA-repair processes (reviewed by Haglund & Dikic, 2005; Hayden & Gosh, 2008). Proteins modified with Lys 63-linked chains are recognized by numerous UBDs (see above), mediating interactions essential for NF-κB activation in response to IL-1 and TNFα stimulation. This topic has been already discussed in detailed in several excellent recent reviews (for example, Hayden & Gosh, 2008).

In DNA-damage response pathways, Lys 63-linked Ub chains seem to have important functions during the recruitment of repair machineries to the sites of DNA damage. The RING-type E3-ligase BRCA1 forms a multimeric protein complex required for the repair of double-stranded DNA breaks (Bennett & Harper, 2008). As BRCA1 is not a Ub-binding protein, the recruitment of this complex to the sites of damage depends on the receptor-associated protein 80 (RAP80), which contains two UIM domains that preferentially bind to Lys 63-linked Ub chains on histones H2A and H2AX (Bennett & Harper, 2008).

Lys 29/Lys 33-linked mixed chains have been recently implicated in the regulation of AMP-activated protein kinase (AMPK)-related kinases. AMPK family member 5 (ARK5/NUAK1) and MAP/microtubule-affinity-regulating kinase (MARK) 4 kinases are polyubiquitinated *in vivo* through Lys 29/Lys 33-coupled chains (Al-Hakim *et al*, 2008). This event blocks their kinase activation by interfering with phosphorylation of the activation-loop residues (Al-Hakim *et al*, 2008). In addition, Lys 29-linked Ub chains formed by the E3 ligase

ITCH/AIP4 have been implicated in the lysosomal degradation of proteins (Chastagner *et al*, 2006).

The formation of mixed-linkage Ub chains is implicated in the activation of the Polycomb protein Ring1B Ub ligase complex (Ben-Saadon *et al*, 2006). Ring1B forms a complex with BMI1, a protein with no detectable ubiquitinating activity. However, BMI1 stimulates the monoubiquitinating activity of the Ring1B ligase complex towards histone H2A. Ring1B catalyses its auto-ubiquitination through atypical Ub chains that involve Lys 6-, Lys 27- and Lys 48-linkage types on the same Ub molecule; this modification does not lead to the degradation of Ring1B, but is instead essential for the efficient monoubiquitination of histone H2A *in vitro* (Ben-Saadon *et al*, 2006).

Heterologous chains—between Ub and Ubl modifiers—also exist and seem to have important physiological roles. A recent study has shown that RING finger protein 4 (RNF4), an E3 Ub ligase that contains several SUMO-interacting motifs, can specifically target SUMOylated promyelocytic leukaemia (PML) proteins for ubiquitination, consequently generating SUMO-Ub heterologous chains (Fig 1D; Tatham *et al*, 2008). This leads to the recognition and recruitment of SUMOylated PML proteins for degradation in the proteasome (Lallemand-Breitenbach *et al*, 2008; Tatham *et al*, 2008). The formation of such heterologous chains is of clinical relevance as treatment with arsenic trioxide triggers SUMO-dependent polyubiquitination and degradation of the PML-RARα fusion protein that causes acute promyelocytic leukaemia (APL; Lallemand-Breitenbach *et al*, 2008; Tatham *et al*, 2008).

Conclusion and future challenges

Atypical Ub chains represent new, albeit poorly understood, molecular signals. They can form a sizeable range of modifications through variations in their length and linkage type. Although experimental data have indicated the existence of a large variety of atypical chains *in vivo*, there is, however, only sparse physiological evidence about their functional role in the cell (Sidebar A). Ongoing work in this area seeks to develop a more complete understanding of the structure and function of polyubiquitin chains, and to define the role and specificity of Ub-binding proteins. An important issue to resolve will be to discriminate atypical Ub chains that encode a valid physiological signal from the ones that are formed during the ubiquitination reaction but that lack any specific biological role. Moreover, it will be important to reveal molecular and atomic details of how each of the various chains is synthesized and attached to substrates, and what determines the specificity of their interactions with Ub-binding proteins *in vivo*. The most challenging task will be to solve three-dimensional structures of a ubiquitinated substrate in complex with a Ub-binding protein. As more studies combine the analysis of biological mechanisms with biophysical and structural studies, future prospects in the field of atypical Ub chains are both exciting and promising.

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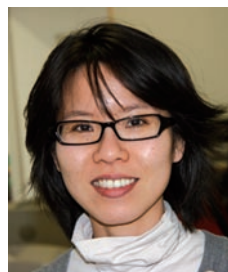Al-Hakim AK, Zagorska A, Chapman L, Deak M, Peggie M, Alessi DR (2008) Control of AMPK-related kinases by USP9X and atypical Lys 29/Lys 33-linked polyubiquitin chains. *Biochem J* **411:** 249–260

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